RESEARCH ARTICLE

Aberrant Epigenetic Alteration in Eca9706 Cells Modulated by Nanoliposomal Quercetin Combined with Butyrate Mediated via Epigenetic-NF-κB Signaling

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Abstract

Since the epigenetic alteration in tumor cells can be reversed by the dietary polyphenol quercetin (Q) or butyrate (B) with chemopreventive activity, suggesting that Q or B can be used for chemopreventive as well as therapeutic agent against tumors. In this study the polyphenol flavonoid quercetin (Q) or sodium butyrate (B) suppressed human esophageal 9706 cancer cell growth in dose dependent manner, and Q combined with B (Q+B) could further inhibit Eca9706 cell proliferation than that induced by Q or B alone, compared with untreated control group (C) in MTT assay. The reverse expressions of global DNMT1, NF-κBp65, HDAC1 and Cyclin D1 were down-regulated, while expressions of caspase-3 and p16INK4α were up-regulated, compared with the C group in immunoblotting; the down-regulated HDAC1-IR (-immunoreactivity) with nuclear translocation, and up-regulated E-cadherin-IR demonstrated in immunocytochemistry treated by Q or B, and Q+B also displayed further negatively and positively modulated effects compared with C group. The order of methylation specific (MS) PCR of p16INK4α: C>B/Q>Q+B group, while the order of E-cadherin expression level was contrary, Q+B>Q/B>C group. Thus, Q/B, especially Q+B display reverse effect targeting both altered DNA methylation and histone acetylation, acting as histone deacetylase inhibitor mediated via epigenetic-NF-κB cascade signaling.

Keywords: Reversible epigenetic modulation - quercetin - butyrate - epigenetic-NF-κB signaling - eca9706 cell line

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Introduction

There is close relationship between neoplasia and inflammation. Many cancers arise from sites of chronic inflammation or irritation, and an oncogenic change always induces an inflammatory microenvironment to promote development of tumors. Inflammatory conditions in selected organs increase the risk of cancer, and in the tumor microenvironment smoldering inflammation contributes to proliferation and cancer progression. Natural bioactive compounds exhibit anti-inflammatory modulation (Pan et al., 2009; Gupta et al., 2011). The dietary flavonoid quercetin and resveratrol have nonsteroidal anti-inflammatory activity that may have applications for anti-inflammation treatment (Donnelly et al., 2004; Tuñón et al., 2009). Our other experiment indicates that the pro-inflammatory cytokine IP-10 in co-cultured human esophageal cancer 9706 cells can be inhibited by the polyphenol quercetin. Prevention of cancer has to be associated with prevention of inflammation, especially chronic inflammation.

Flavonoid quercetin is one kind of yellowish powdered crystalline compound, (C15H10O7 in structure with five OH groups), widespread in vegetables, fruits and some beverages. It also is one kind of chemopreventive agent with both anti-neoplasm and anti-inflammation effects. Earlier report about the activity of protein kinase C and tyrosine kinase could be inhibited by quercetin in HL-60 cells (Kang et al., 1997). Our previous experiment showed that the natural polyphenol quercetin could arrest cancer cell cycle progression in G2-M phase and induce cells toward apoptosis in Eca109 cells (Zhang et al., 2007). Further research indicates that the tea polyphenols offer cancer prevention against lipid oxidation or adhesion molecule expression (Pandurangan et al., 2012; Zheng et al., 2012). The genetic mutations along with epigenetic alterations, mainly including DNA promoter hypermethylation of tumor suppressor genes, aberrant histone acetylation modification or micro RNAs, can be found in the cancer cells. The epigenetic alteration is reversible under certain condition induced by the natural polyphenols, which can be used for therapy as well as chemoprevention agents (Link et al., 2010). The quercetin can exert anti-tumor properties mediated via demethylation of p16INK4α gene promoter (Tan et al., 2009). When quercetin combined with trichostatin A (TSA, histone deacytylase (HDAC) inhibitor), they cooperatively kill human leukemia cells (Chen et al, 2005).

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Butyrate is a short chain fatty acid ($CH_3CH_2CH_2COOH$) generated from dietary fiber in body. There are lots of papers about the butyrate against various kinds of tumor cells, including cancer prevention as well as anti-cancer treatment, acting as HDAC inhibitor (HDI). The oral cancer cell proliferation can be inhibited by 2 mM -3 mM sodium butyrate (NaBu), while 1 mM NaBu displays no inhibitory effect (Miki et al., 2007). HDI butyrate can cause G2 cell-cycle arrest and up-regulation of p21/waf1/cip1 protein, and also can activate the p53 protein via hyper-acetylation and nuclear re-localization in neuroblastoma cells (Condorelli et al., 2008). The butyrate can induce apoptosis through JNK MAPK activation in colon cancer RKO cells (Zhang et al., 2010). A butyrate derivative combined with doxorubincin may be beneficial in future therapy of HL-60 cells (Abizadeh et al., 2001). The plant phonolic compounds combined with sodium butyrate can effectively induce anti-tumour activity (Indap et al., 2003). Combined agents contribute to anti-tumor therapy and chemoprevention, especially combined with natural agent. However, So far, the information about the chemoprenventive effect induced by polyphenol quercetin combined with butyrate has been limited. We prepared nanoliposomal quercetin (nLQ/Q) to make it dissoluble into PBS, since the quercetin is hardly dissoluble into water. We designed to explore whether the reversibility of aberrant epigenetic modification induced by Q combined with B would be further promoted than Q or B alone in human esophageal cancer Eca9706 cells.

Materials and Methods

Preparation of nanoliposomal quercetin and sodium butyrate

The quercetin powder (Sigma US), cholesterol, lecithin, encephalin and polyglycol-4000 in ratio of 6:4:9:5:1 were dissolved in chloroform and methanol (3:1) which was processed by revolutionary-evaporator under vacuum for 4-8h, finally dissolved into PBS. The liposomal quercetin was cleaved by Sonics repeatedly until passing through a filter membrane with 80 nm pore size (Sigma, USA) to prepare the nanoliposomal quercetin (Q) in 4.8mg/ml in PBS as stocking solution. The water soluble powder of sodium butyrate (B) was purchased from Sigma USA and 0.227 mol/L butyrate dissolved in PBS as stocking solution.

MTT assay

After each group of Eca9706 cells, including Q group (20 μM, 40 μM 60 μM), B group (1 mM, 2 mM, 4 mM), Q+B group (40 μM Q+2 mM B) and no drug control group cultivated into repeated 5 wells of one 96-well plate (Corning, USA) for 48h, 5 mg/ml MTT (Amersco, USA) in 20 μl was added and re-incubated for 4h. The supernatant was removed, followed by recruitment of 150μl DMSO to dissolve the formazone product, and OD value in each well was determined by Label Sensors (Sunrise, China) under 492nm wave length. We collected each group of Eca9706 cells for MTT assay after 48h incubation, since floating dead cells appeared apparently after incubation for 72h and 48h incubation growth potential was superior over 24h observed under inverted microscope.

Grouping of cultured Eca9706 cells

The Eca9706 cancer cells were cultured with DMEM medium plus 10% FBS for 48h routinely. The cultured Eca9706 cells (1×10^7)were divided into 4 groups based on the detected MTT assay: 1 Q group: with 40 μmol/L Q; 2 B group: cultivated with 2mmol/L sodium butyrate; 3Q+B group, cultivated with 40 μmol/L Q combined with 2mmol/L B (doses with 27%-28% growth suppressing rates) and 4 C group: cultured for 48h with neither Q nor B.

Immunoblotting of Eca9706 cells

The total protein was extracted from each group of 9706 cells by using CelLyticTM Cell Lysis Reagent (Sigma, USA), and the protein concentration was detected with Bradford method. The performed SDS-PAGE gel was electro-transferred onto the pre-treated nitrocellulose membrane (NCM, Protran, USA). The primary antibodies, including anti-DNMT1 (polyclonal, Boisynthesis, China), anti-HDAC1 (polyclonal, Santa Cruz, USA), anti-NF-κBp65 (monoclonal, Santa Cruz, USA), anti-cyclin D1 (monoclonal, Zymed, USA), anti-caspase-3 (polyclonal, Zymed, USA) and anti-β-actin (Santa Cruz, USA) was added into each lane as loading control. The primary anti-sera were incubated with corresponding NCM on a vibrator under room temperature for 2.5 h. The TTBS buffer (0.15 mol/L NaCl and 0.05% Tween 20 added into 0.1 mol/L Tris-Cl buffer, pH 7.5) was used as blocking or washing solution. The goat or mouse IgG-HRP (monoclonal ZSGB, China) was used as the secondary antibody to incubate the NCM on a vibrator under room temperature for 1.5h, finally the brownish color developed on the NCM under DAB substrate for 20min. The grey scaled means (GSM) of target bands were determined by an Image-Scanner (ShanFu, China).

Methylation specific PCR (MSP) of p16INK4a gene promoter

The genome DNA was extracted from each group of Eca9706 cells by using a kit (Tiangen, China). The procedures from cell lysis to DNA eluted from column filter were performed according to the instruction. After the DNA purity and concentration was determined, the MSP was performed by using Methylamp™ DNA Modification Kit (Epigentek, USA) strictly according to user guide. Firstly the DNA is chemically denatured to allow bisulfite reagent to react specifically with single-stranded DNA to convert C into U. The modified DNA captured onto a column filter was collected through cleaning and elution. The methylation-specific primers for p16INK4α promoter included forward primer, 5' -ttattagagggtggggcggatcgc-3' and reverse primer, 5' -gaccccgaaccgcgaccgtaa-3'. The real time MS-PCR procedures for p16INK4α were performed as follows: 95°C 5min, 95°C 50s 35cycles, 60 °C 50s 35cycles, 72°C 50s 35cycles, 72°C 5min. The DNA 100 ladder marker (Solarbio Biotech, China) was used in 2% agarose gel electrophoresis and the MSP results were analyzed by Image-Scanner (ShanFu, China).

Table 1. Growth Suppressing Rates (GSR ±s) Detected by MTT Assay in Eca9706 Cells Treated with Q/B or Q+B, Compared to Control Group

Quercetin (Q), butyrate (B)	OD value	GSR%
20μM Q	0.696±0.030	12±0.01
40μM Q	0.571±0.030	28±0.01*
60μM Q	0.485±0.014	39±0.06
1 mM B	0.683±0.030	14±0.01
2 mM B	0.577±0.037	27±0.01#
4 mM B	0.515±0.038	35±0.01
40μM Q+2 mM B	0.475±0.036	40±0.01**
C group (C)	0.792±0.021	

**vs C, P<0.008, **vs */#P<0.008

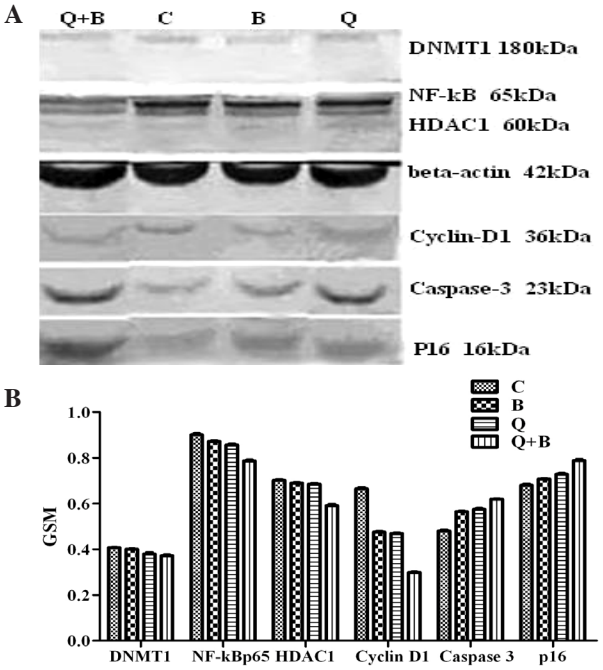

Figure 1. A. Immunoblotting Showed Down-regulated Expressions of DNMT1, NF-κBp65, HDAC1, and Cyclin D1 and Up-regulated Expressions of Caspase-3 and p16INK4α in Eca9706 Cells, Q+B> Q/B, Compared with C Group, P<0.008; B. Diagram Showed Decreased Global DNMT1, NF-κBp65, HDAC1, Cyclin D1 and Increased Caspase 3, p16INK4α Levels in Eca9706 Cells Treated by Q/B or Q+B Compared with C. The Increased/decreased Sequence was Q+B>Q/B>C, P<0.05

Immunocytochemical reactivity of E-cadherin and HDAC1

The E-cadherin antiserum (monoclonal, Zemed, USA) or HDAC1 antiserum was used as primary antibody, the SP kit from ZhongShan (China) was used as secondary and tertiary anti-sera. The primary antibody replaced by PBS was performed as the negative control. The grey scale means (GSM) for target bands were determined by an Image-Scanner (ShanFu, China).

TUNEL assay

The assay was performed according to the user guide from Promega USA. The TdT buffer substituted for TdT enzyme was performed as the negative control. The apoptotic cells with bluish-violet apoptotic signals were enumerated per two hundred Eca9706 cells in each group as the apoptotic rate.

Statistical analysis

Statistical analyses were performed by use of SPSS 12.0 statistical package. The data shown as (mean ±s) represented at least three independent experiments. The difference was analyzed with one way ANOV, P<0.05 as significant level; while the difference in complex parameters was analyzed with LSD-t test, P<0.008 was considered as significant level.

Table 2. GSM ($\bar{\chi}$±s) of DNMT1, NF-κBp65, HDAC1, Cyclin D1, Caspase-3 and p16INK4α of Immunoblotting in Eca9706 Cells Treated with Q/B or Q+B, Compared to Control Group

Index/group	Control (C)	B
DNMT1	0.407±0.001	0.401±0.001&
NF-κBp65	0.901±0.005	0.872±0.003&
HDAC1	0.702±0.003	0.690±0.002&
Cyclin D1	0.665±0.005	0.475±0.003&
Caspase 3	0.480±0.005	0.565±0.002&
P16 INK4α	0.680±0.005	0.708±0.003&

*/&/** vs C, P<0.008; ** vs */&P<0.008

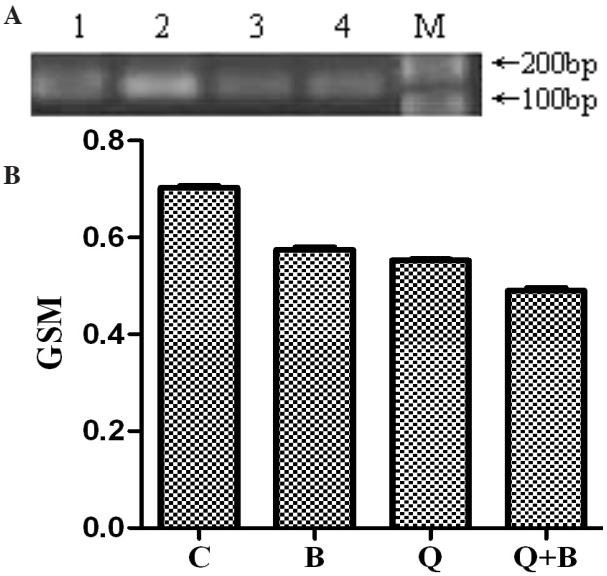

Figure 2. A. p16INK4α MS-PCR in Each Group of Eca9706 Cells; Lane 1: Q Group; Lane 2: C Group; Lane 3: Q+B Group; Lane 4: B Group; M Lane: DNA Marker. The Methylated p16 INK4α Located at 150bp. B. Diagram of Methylated p16INK4α in Each Group of Eca9706 Cells. Diagram of p16INK4α MS-PCR Showed That the MSP Order was C>B/Q>Q+B, P<0.05

Results

MTT assay

The MTT assay showed that Q or B suppressed cell growth in dose dependent manner, Q+B further inhibited the cell growth compared with Q/B or C group, P<0.008. However, there was no significant difference in suppressing cell growth between Q and B, P>0.05 (Table 1). The growth suppressing rate (GSR) = 1-experimental OD value/ control OD value×100

Immunoblotting

The immunobkotting showed that the expressions of global DNMT1, NF-κBp65, HDAC1, and cyclin D1 were down-regulated by Q or B, and further by Q+B, compared with C, Q or B, P<0.008. However, the expressions of caspase-3 and p16INK4α were up-regulated, and further

Table 3. HDAC1-IR Location Pattern, GSM ($\bar{\chi}$±s) of E-cadherin-IR and Apoptotic Rates in Eca9706 Cells Treated with Q/B or Q+B, Compared to Control Group

Index/group	Control (C)	B
HDAC1-IR location	90-95% cells in nuclei	85% cells in cytoplasm
E-cadherin-IR	90.92±5.56	107.96±1.06$^{\&}$
Apoptotic cells	5.0± 0.5%	38 ± 0.5%$^{\&}$

$^{*/\&/**}$vs. C, P<0.008; **vs.$^{*/\&}P < 0.008$; *vs. $^{\&}P > 0.05$

by Q+B compared with C, Q or B, P<0.008 (Table 2, Figure 1).

MSP of p16INK4α gene promoter

In MSP of DNA genome of p16INK4α located at 150 bp, the GSM (±s) of methylated p16INK4α were C group (0.703±0.004)>B group (0.575±0.005) /Q group (0.553±0.003)>Q+B group (0.490±0.006), P<0.05 (Figure 2A, Figure 2B). The order of p16INK4α methylation in Eca9706 cell groups was C>B/Q>Q+B.

HDAC1-immunocytochemical reactivity (IR) and E-cadherin-IR

The HDAC1-IR as fine brownish granules mostly located in the nuclei of 90%-95% Eca9706 cells showed nuclear pattern in C group; while 85%-90% Eca9706 cells showed cytoplasmic/mixed pattern after treated with Q/B or Q+B, P<0.008. E-cadherin–IR appeared as fine brownish granules located over the intact Eca9706 cell surface. The GSM order of E-cadherin was Q+B (120.98±2.15) >Q (110.90±1.31) or B (107.96±1.06)>C (90.92±5.56), P<0.05 (Table 3, Figure 3).

Apoptosis detected by TUNEL assay

The bluish-violet apoptotic signals were mainly located in Eca9706 cell periphery. The order of apoptotic rates was Q+B:56±0.5% >Q:42±0.5% /B:38±0.5% >C:5±0.5%, P<0.05 (Table 3, Figure 3).

Discussion

NF-κB is activated by response to various stimuli, such as viral or bacterial infection, oxidant stress, immune response and carcinogenesis. Transcription factor NF-κB is a common final target for many cell proliferation and inflammatory pathways (Wilczynsk et al., 2011). The activated NF-κBp65 (RelA) and the aberrant HDAC activity play the pivotal role of tumorigenesis. There is link between RelA/p65 and class I HDACs in nuclear translocation as well as RelA/p65 DNA binding activity which can be markedly diminished by HDAC inhibitor (Lehmann et al., 2009). In prostate cancer cells the apoptosis is induced through inactivation of nuclear factor–kappaB and activation of caspase pathway after anti-cancer treatment (Park et al., 2011). Butyrate induces human colon cancer RKO cell apoptosis through activation of caspase pathway (Zhang et al., 2010). In present experiment the down-regulated HDAC1 with altered nuclear translocation in treated groups to

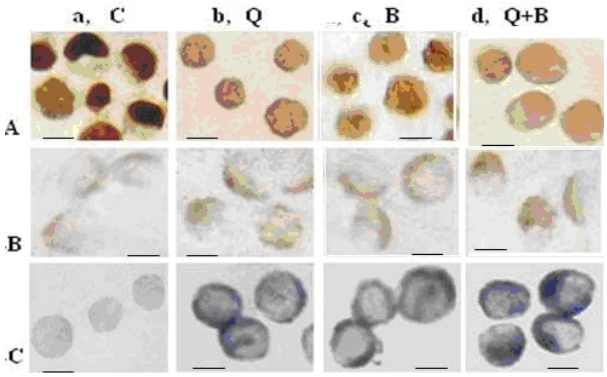

Figure 3. Aa-d, Representative HDAC1-IR; Figure 3Ba-d, representative E-cadherin-IR; Figure 3Ca-d, representative TUNEL signals in each group of Eca9706 cells. Figure 3a-d, HDAC1-IR: C>Q/B>Q+B; E-Cadherin-IR: Q+B>Q/B>C; TUNEL signals: Q+B>Q/B>C. The __ bar represents 10 μm. 1000×

inhibit Rel A/p65 activity, and down-regulated HDAC1, Rel A and cyclin D1, and up-regulated caspase-3 and p16INK4α, the growth suppressing rates (GSR) were induced by Q/B and further by Q+B, acting as HDAC inhibitor via HDAC-NF-κB cascade signaling. Besides, the up-regulated expression of p16INK4α by Q/B and further by Q+B in immunoblotting was associated with the local hypermethylation of p16INK4α gene promoter attenuated by Q/B and further by Q+B in MS-PCR. The NF-κBp65 activity and HDAC are important inducers of epithelial-mesenchymal transition (EMT), cell metastasis and invasiveness (Strippoli et al., 2010; Lei et al., 2010). In this experiment the E-cadherin expression was increased, suggesting that the reversal EMT may be contributed to inhibiting migration/invasive potency of Eca9706 cells mediated via HD1 or inactivation of NF-κBp65 induced by Q/B, especially by Q+B. Thus, the promoted anti-cancer effect by Q/B and further by Q+B may be mediated through targeting both altered DNA methylation and histone acetylation, mediated via epigenetic-NF-κB-signaling. Flavonoids derived from fruits, vegetables, etc, can suppress the proinflammatory cell signaling (such as activated NF-κB) pathways and thus can prevent and even treat the cancer (Prasad et al., 2010).

Screening of potential cancer chemopreventive agents mainly based on anti-oxidant/anti-inflammatory mechanism in murine hepatoma cell cultures was induced by sodium butyrate or polyphenols, including quercetin, EGCG, resveratrol, curcumin, etc. (Gerhauser et al., 2003). The polyphenol quercetin may prevent cardiovascular disease due to its anti-oxidative stress; the healthy male subjects consumed 150 mg/d quercetin for 8 wks intermitted by a three-week washout phase (Pfeuffer, 2013). The cancer chemopreventive experiment on the Wistar rat hepatocarcinogenesis model treated with histone deacetylase inhibitor, tributyrin (butyric prodrug, 2g/kg body wt) daily for 8 wks resulted in lower HDAC activity and Hdac3 and Hdac4 gene expression, and etc (de Corti, 2013). Moreover, Steliou k et al indicate that the histone deacetylase inhibitor butyrate is not only capable to suppress cancer growth, but also to regulate other medical disorders, such as neurological,

hematological and insulin resistant obesity in mouse models. Simultaneously they discuss the butyrate shortcoming, including multigram doses and short half life; e.g., the butyrate used in mM level first-passes hepatic clearance with short half life (Steliou et al., 2013). On the contrary, the nanoliposomal quercetin with prolonged half life (Jun et al., 2012) is commonly used in μM level. When the butyrate combined with polyphenol quercetin used for disease chemoprevention or treatment, not only the combined effect is promoted markedly, but also the butyrate shortcoming may be overcome to certain extent and with less economic reckoning. Since different cell lines in sensibility to Q or B may be different, it had better to detect the target cell line in vitro prior to applying chemopreventive agent Q or B to it in vivo.

About epigenetic therapeutic strategy the DNA demethylation agent combined with HDI have been shown to produce synergistic reactivation of anti-tumor effects to yield promising results in clinical studies (Kihslinger et al., 2007). Both quercetin and butyrate themselves exhibited combined effectiveness of demethylation and HDI in anti- human esophageal cancer 9706 cells. Thus, the quercetin and sodium butyrate themselves exhibit promising chemopreventive as well as therapeutic strategy merit to be further studied.

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